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Dynamic Chromatin Environment of Key Lytic Cycle Regulatory Regions of the Epstein-Barr Virus Genome

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The ability of Epstein-Barr virus (EBV) to establish latency allows it to evade the immune system and to persist for the lifetime of its host; one distinguishing characteristic is the lack of transcription of the majority of viral genes. Entry into the lytic cycle is coordinated by the viral transcription factor, Zta (BZLF1, ZEBRA, and EB1), and downstream effectors, while viral genome replication requires the concerted action of Zta and six other viral proteins at the origins of lytic replication. We explored the chromatin context at key EBV lytic cycle promoters (*BZLF1*, *BRLF1*, *BMRF1*, and *BALF5*) and the origins of lytic replication during latency and lytic replication. We show that a repressive heterochromatin-like environment (trimethylation of histone H3 at lysine 9 [H3K9me3] and lysine 27 [H3K27me3]), which blocks the interaction of some transcription factors with DNA, encompasses the key early lytic regulatory regions. Epigenetic silencing of the EBV genome is also imposed by DNA methylation during latency. The chromatin environment changes during the lytic cycle with activation of histones H3, H4, and H2AX occurring at both the origins of replication and at the key lytic regulatory elements. We propose that Zta is able to reverse the effects of latency-associated repressive chromatin at EBV early lytic promoters by interacting with Zta response elements within the H3K9me3-associated chromatin and demonstrate that these interactions occur *in vivo*. Since the interaction of Zta with DNA is not inhibited by DNA methylation, it is clear that Zta uses two routes to overcome epigenetic silencing of its genome.

Epstein-Barr virus (EBV) exists in a highly restricted form of latency in Burkitt's lymphoma (BL) (39). The majority of viral genes, including approximately 60 lytic cycle-associated genes, are not expressed (27); indeed, the effective silencing of these genes contributes to the ability of infected cells to evade the immune system and thus survive and cause disease (27).

Despite their highly restricted viral gene expression pattern BL cells initiate lytic replication rapidly following stimulation of the B-cell receptor (surface IgG) (46, 47). The expression of EBV lytic cycle genes is coordinated by Zta (BZLF1, ZEBRA, EB1, and Z), the product of the *BZLF1* gene (31, 43, 45). Zta is a DNA-binding sequence-specific transcription factor known to activate several EBV lytic cycle genes, including the *BRLF1* gene which encodes a second important transcription factor Rta (41, 42). Zta directly interacts with several lytic promoters and with the origins of lytic replication (oriLytL and oriLytR) *in vitro* (41) and *in vivo* (2, 9, 52) through a direct interaction with Zta-response elements (ZREs). Zta displays the unusual feature of interacting with some ZREs only when they are methylated (2–4, 9, 26). Thus, host-driven methylation of the EBV genome in latency engenders a repressive environment for gene expression and yet primes the methylated ZREs in lytic cycle promoters to be responsive to Zta (14, 25). In addition, Zta is a replication factor for the EBV genome; it is required to assemble the six viral components of the replication machinery at the origin of lytic replication (15, 16).

Regulation of *BZLF1* transcription is therefore crucial, both to constrain the expression of Zta protein during latency and to facilitate its activation when required. In BL cells, the host transcriptional repressors ZEB and MEF2 play roles in latency, repressing transcription of *BZLF1* (5, 11, 22, 28). Transcription from the BZLF1 and BRLF1 promoters is activated in response to signal transduction arising from stimulation of the B-cell receptor and then amplified through Zta autoactivation (18, 33, 51). In contrast, transcription of the other EBV early lytic genes occurs less directly following stimulation of the B-cell receptor, with a strict dependence on protein synthesis (18, 33).

The EBV genome consists of double stranded DNA that resides in the nucleus, where it is associated with histones and presumably subject to the same chromatin/histone modifications as the host genome (36). Silencing of viral lytic cycle gene expression could be simply explained by the absence of an open chromatin environment at lytic cycle promoters. The potential contribution of differential histone modifications, particularly acetylation of histones H3 and H4 to the regulation of EBV lytic cycle has been addressed, providing support for opening up of the chromatin environment during the lytic cycle (6, 7, 22–24, 32, 51). However, Countryman et al. discovered that this model is too simplistic to account for the silencing of lytic promoters during latency by demonstrating that reprogramming histone acetylation is not sufficient to activate the EBV lytic cycle in all cell types (7, 8).

Posttranslational modification of the histone 2A variant, H2AX, has also been associated with EBV lytic cycle; H2AX becomes phosphorylated during lytic replication (29). Phosphorylation of H2AX is a common theme among gammaherpesviruses, with a conserved viral protein kinase directing phosphorylation of H2AX for both EBV (*BGLF4*) and MHV68 (*orf36*) (48). H2AX is a component of the DNA damage response, known to be activated during EBV replication, and in some cell types H2AX is required for the replication of MHV68 (50).

Despite the focus of attention on regulation of the *BZLF1* pro-

Received 20 September 2011 **Accepted** 10 November 2011

Published ahead of print 16 November 2011

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doi:10.1128/JVI.06334-11

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TABLE 1 Primer sequences[a]

EBV gene target[b]	Additional EBV target	QPCR primer for ChIP (5′–3′)
BALF5-F		GATCGTGATAGCGTCTTCTGC
BALF5-R		GCAACATGCCTCTGGTGA
BMRF1-1F		GATTGGACTTCACACCAGGAA
BMRF1-1R		TCACAATGCGTTCAGAGAGG
BMRF1-2F		CACTGCGGTGGAGGTAGAG
BMRF1-2R		GGTGGTGTGCCATACAAGG
BMRF1-3F		CACCATGCTGGTGGTAGATG
BMRF1-3R		GCATGGTCATAGCACTTGGA
BMRF1-4F		CTGAGGAACGAGCAGATGATT
BMRF1-4R		CGTAGAGATCCGGATTGAGTG
OL1-F (Flank Orilyt L)		GCGCAACAGTGCCACCAACC
OL1-R		CAGGACCTGGCGGTAGTGCAG
OL2-F		GCTCCACTGCACCTGGAAT
OL2-R		CCAGAGGAGCCCCAGAAC
OL3-F	OR3-F	CTCTTTTTGGGGTCTCTGTG
OL3-R	OR3-R	CCTCCTCCTCTCGTTATCC
OL4-F		CTAGAGGTCCGCGAGATTTG
OL4-R		ACCTCTAGGCTCCACCCACT
OL5-F	OR4-F	CAGCTGACCGATGCTCGCCA
OL5-R	OR4-R	ATGGTGAGGCAGGCAAGGCG
OL6-F		GGCCTGAAGAGGTTGACAAG
OL6-R		GAGGTAAGCCGTTCCAGATG
OL7-F		GGTACCCTGCATCCTGTGTT
OL7-R		GCGGAGAGGTGTTTCTCTTG
OL8-F		CATGACAGCCAATCCAACAC
OL8-R		CTGCGCCTACAGATCATCAA
OR1-F (Flank Orilyt R)		CCGCATGTCCAACCACCACG
OR1-R		ATGCTACCTAGGCCTGCGTCC
OR2-F		TTCCATTATCCTGGAGGTATCC
OR2-R		GCTGAATCCTACCTAGCTCCAC
OR5-F		GCTGGTTAAGCTGACGACCT
OR5-R		AGACATGCAGGAACACATGG
OR6-F		AGCCGAGCAGATTCTAATGG
OR6-R		CAACAGGTGTGCAGGTGTG
OR7-F		GGACGCAGCTACTTGACCTT
OR7-R		AGTGGACGCAGCACTTATCA
RP1-F		AGGTCCTCCTCTGGACTGTG
RP1-R		TTGATCTCGAGGTGCAGAAG
RP2-F		CCTGTTGTTTCGGAGAATGG
RP2-R		AATTTACAGCCGGGAGTGTG
RP3-F		GGCTGACATGGATTACTGGTC
RP3-R		TGATGCAGAGTCGCCTAATG
RP4-F		TCGCGATGCTATAAACCAGA
RP4-R		AGGGCATTCCATAAAGCAAA
RP5-F		CGGATGTCCAGAGTGCCTA
RP5-R		TGGACCAACATGTTCAGGAG
RP6-F		GTGATGAGGACGAGGATGGT
RP6-R		CCTCGTCAGACATGATTCACA
ZP1-F		CACGGCCATGCTATCTTGTA
ZP1-R		TGCCACTGGTCTCATCCTC
ZP2-F		GGAGGAATGCGATTCTGAAC
ZP2-R		CTGACCTCACGGTAGTGCTG
ZP3-F		AAGGCCAGCTAACTGCCTATC
ZP3-R		ACAGCTGAGGTGCTGCATAA
ZP4-F		GAGCCACAGGCATTGCTAA
ZP4-R		ACCAGCCTCCTCTGTGATGT
ZP5-F		CATGCAGCAGACATTCATCA
ZP5-R		GACGAACTGACCACAACACTAGA
ZP6-F		TGTCCACATATGGCTGCTTC
ZP6-R		GCAAGTCATCTGTTGGAGGAC
ZP7-F		CGCTCACGTAGCTCCTCTG
BNRF1-F		TGTGACACCAACAGGTGTTGCCTTG
BNRF1-R		ACCCCAAAGAGGGCAAAGCCTAC
BCRF1-f		GGGAGGTACATGTCCCCCAGCATT
BCRF1-R		CTGTGGACTGCAACACAACATTGCC
QP-F†		CTGTCACCACCTCCCTGATA
QP-R†		GAACACTCCCTCAGTGGTCA
BGLOB-F*		GGCAACCCTAAGGTGAAGGC
BGLOB-R*		GGTGAGCCAGGCCATCACTA
GAPDH-F†		CTCATGCCTTCTTGCCTCTT
GAPDH-R†		TTGATGGCAACAATATCCACTT

[a] Forward (F) and reverse (R) primer orientations areindicated in the primer designations.
[b] *, Gallagher et al. (20); †,Palermo et al. (34).

moter, little is known about the chromatin context of the other lytic cycle regulatory elements during latency. We explore here the association of repressive and activating histone modifications at key lytic cycle regulatory elements within the EBV genome during latency. The ability of Zta to interact with each of these regions is investigated together with the chromatin context during the lytic cycle using sequential chromatin immunoprecipitation (ChIP) assays. We propose a model in which the ability of Zta to interact with repressive chromatin combined with its ability to interact with methylated DNA allows it to overturn both strands of the epigenetic silencing of key EBV lytic genes imposed by the host.

MATERIALS AND METHODS

Cell culture and induction of EBV lytic replication. Group I EBV-positive Akata BL cells (46) were maintained in RPMI medium supplemented with 10% (vol/vol) fetal bovine serum, 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 2 mM L-glutamine (Invitrogen) at 37°C with 5% CO_2. For EBV lytic induction, cells were seeded in log-phase growth at 5×10^5cells/ml. After 24 h, the cells were concentrated to 2×10^6 cells/ml and treated with 0.125% rabbit anti-human IgG (Dako) or Dulbecco phosphate-buffered saline. The cells were harvested at various time points up to 48 h postinduction. To stall EBV genome replication, cells induced with anti-IgG were treated with 100 μM acyclovir.

Antibodies. Acetyl-histone H3, acetyl-histone H4, and phosphorylated H2AX ser139 (Millipore) and trimethyl histone H3K9 and trimethyl histone H3K27 (Abcam) were used for ChIP and Western blot analysis. Goat polyclonal antibody sc-17503 to Zta (Santa Cruz Biotechnology) was used for the ChIP assays, and BZ1 mouse monoclonal antibody to Zta was used to detect the protein by Western blotting (53); control goat and rabbit immunoglobulin G (IgG) was obtained from Santa Cruz Biotechnology for the ChIP assays. β-Actin (Sigma) was used to detect proteins by Western blotting.

Western blotting. Portions (10 μl) of total cell lysates (2×10^5 cells) or immunoprecipitated proteins were resolved on a 12% Bis-Tris NuPAGE gel in morpholinepropanesulfonic acid buffer (Invitrogen). After SDS-PAGE, the proteins were transferred onto nitrocellulose membranes (Santa Cruz Biotechnology) and incubated with indicated antibodies overnight at 4°C. The following day, the membranes were incubated with horseradish peroxidase-linked secondary antibodies (Amersham), and the proteins were detected by chemiluminescence.

ChIP assay. ChIP experiments used a modified version of the protocol as described by Bark-Jones et al. (1). Briefly, the lysates were sonicated on ice (10 times with 10-s pulses each time, with a 30% amplitude output on a Branson model 250 Microtip at setting 5 [Sonics Vibracell]) to obtain 200- to 600-bp DNA fragments. For immunoprecipitation, 10 μg of an-

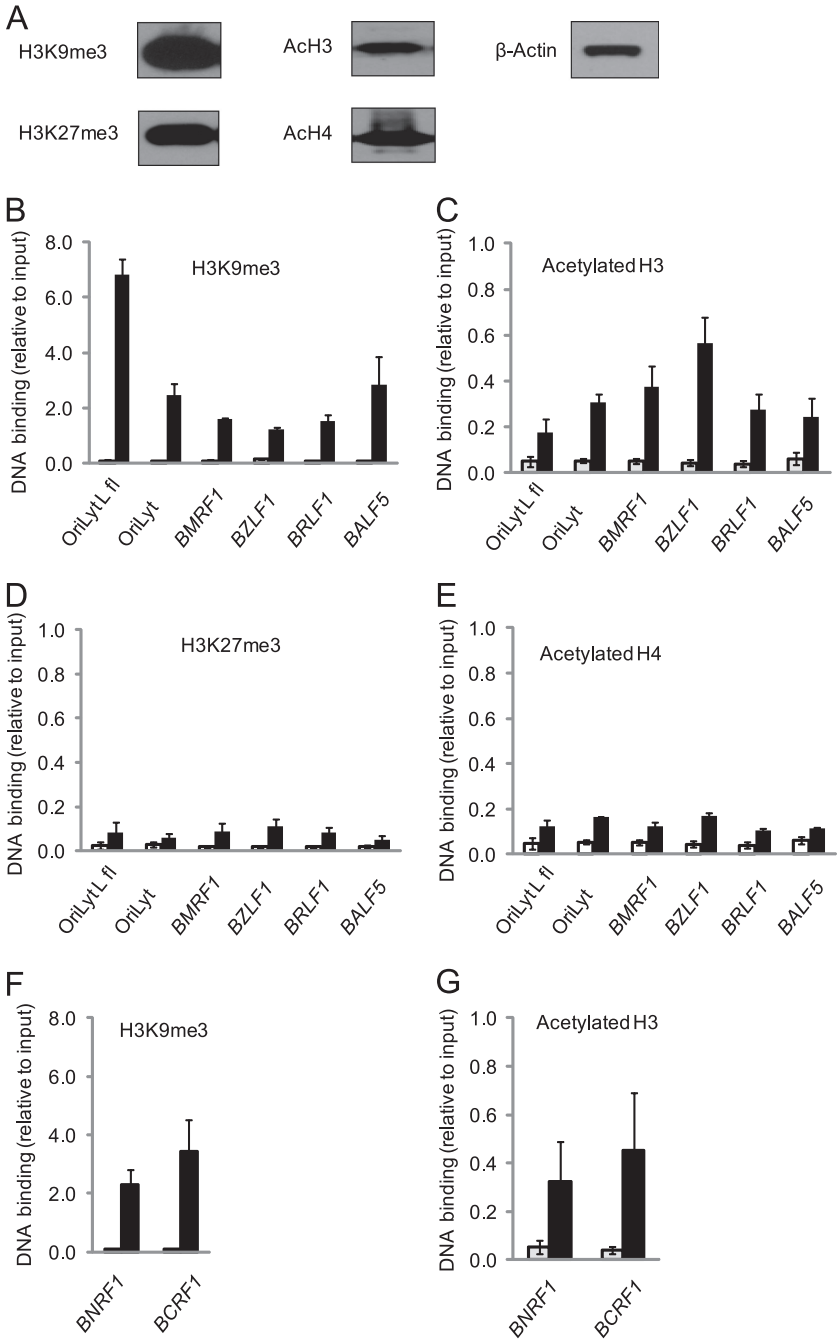

FIG 1 Histone modifications associated with key lytic regulatory regions during latency. Total protein extracts and chromatin were prepared from Akata BL cells. (A) Proteins were fractionated on SDS-polyacrylamide gels and subjected to Western blotting with the indicated antibodies. (B to G) Chromatin was precipitated with the antibodies indicated above each panel, and associated DNA was purified and amplified with primers specific for each of the regulatory regions. The data are from experiments carried out on two independent batches of chromatin. In each case, the gray bars represent the signal from the control antibody, and the black bars represent the signal from the histone modification antibodies. DNA binding is expressed as the percentage of input chromatin. The primers used to amplify each region were OriLytL flank (OL1), OriLyt (OL5), *BMRF1* promoter (BMRF1-2), *BZLF1* promoter (ZP4), *BRLF1* promoter (RP3), *BALF5* promoter (BALF5), and *BNRF1* and *BCRF1*.

tibodies were used with chromatin from 5×10^6 cells. The immune complexes were collected with preblocked 50% protein A/G-Sepharose bead slurry. After stringent washes and protein digestion of the immune complexes, the eluted DNA was purified using gel extraction kit (Qiagen) and eluted in 100 μl of double-distilled H_2O or Tris-EDTA (TE) buffer to be analyzed further by real-time quantitative PCR (QPCR).

The ability of the anti-Zta antibody to precipitate Zta in the ChIP conditions was verified by resuspending the beads after immune precipitation in 25 μl of 2× SDS sample buffer and analyzed by Western blotting.

ChIP-reChIP. Sequential ChIP experiments (ChIP-reChIP) (21) were undertaken using chromatin from 3×10^7 cells and 60 μg of antibodies in

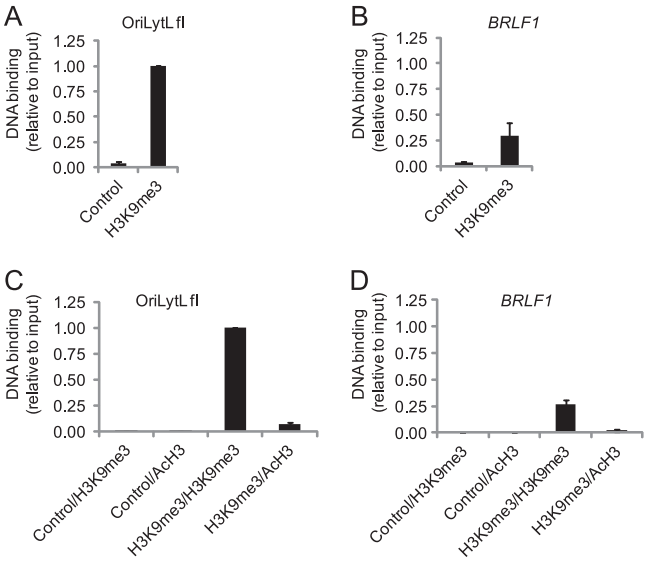

FIG 2 Coassociation of repressive marker of chromatin. Chromatin was prepared from Akata cells. The initial round of ChIP was undertaken with a H3K9me3 or a control antibody as shown below the histogram. The sample was then divided, and some DNA was purified directly and analyzed by PCR, while the remainder of the DNA complexes were eluted and subjected to a second round of precipitation with different antibodies. The data are from experiments carried out on two independent batches of chromatin. (A and B) Chromatin precipitated in the first round was processed to purify DNA, and this was amplified with primers sets specific for regions flanking OriLytL (OL1) (A) or at the *BRLF1* promoter (RP3) (B). The amount of chromatin bound in each case is expressed relative to the binding of H3K9me3 to the flank region of OriLytL (OL1). (C and D) Chromatin precipitated in the second round was processed to purify DNA, and this was amplified with primers sets specific for regions flanking OriLytL (OL1) (C) or at the *BRLF1* promoter (RP3) (D). The data are expressed relative to the binding of H3K9me3 to the flank of OriLytL (OL1).

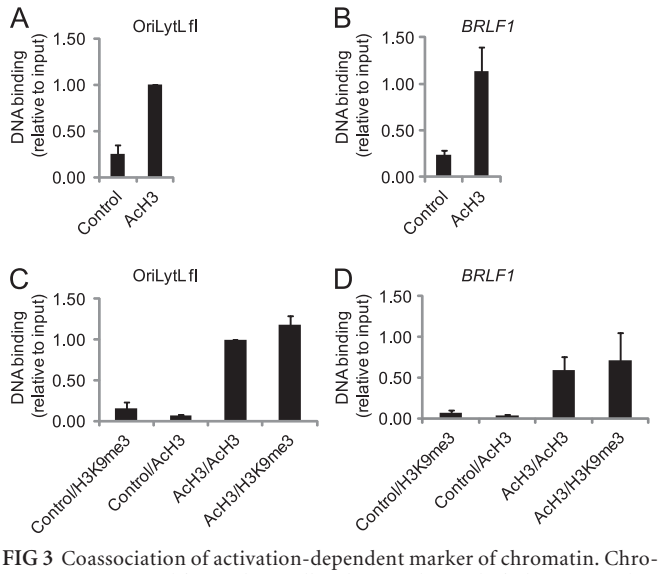

FIG 3 Coassociation of activation-dependent marker of chromatin. Chromatin was prepared from Akata cells. The initial round of ChIP was undertaken with an acetylated H3 or a control antibody. The sample was then divided and some DNA was purified, while for the remainder were eluted and subjected to a second-round of precipitation with different antibodies as indicated below each data set. The data from experiments carried out on two independent batches of chromatin. (A and B) Chromatin precipitated in the first round was processed to purify DNA, and this was amplified with primers sets specific for regions flanking OriLytL (OL1) (A) or at the *BRLF1* promoter (RP3) (B). The amount of chromatin bound in each case is expressed relative to the binding of acetylated H3 to OL1. (C and D) Chromatin precipitated in the second round was processed to purify DNA, and this was amplified with primers sets specific for flanking OriLytL (OL1) (C) or at the *BRLF1* promoter (RP3) (D). The data are expressed relative to the binding of acetylated H3 to OL1.

the first round. The eluted complexes were diluted and subjected to a second round with 10 μg of each antibody. The DNA was then purified using a gel extraction column (Qiagen) and analyzed by PCR.

Real-time PCR analysis. QPCR analyses were undertaken using a Sensimix SYBR kit (Bioline) on an ABI 7500 real-time QPCR system (Applied Biosystems). The absolute quantitation method was used with dilutions of input DNA to generate the standard curve. Primer sets to detect EBV genome regions were designed using Primer3 (40) and are shown in Table 1.

Quantification of EBV genome copy number. A total of 2×10^6 Akata cells were harvested, and DNA was extracted using a Wizard genomic DNA purification kit (Promega). DNA was subjected to QPCR using primers specific to the EBV and human genomes (EBV DNA polymerase gene and the human β-globin gene). The relative amount of EBV genome copies to human genome was analyzed as described previously (20).

RESULTS

Histone modifications associated with key lytic regulatory regions during latency. The use of ChIP to map the chromatin associated with early lytic cycle regulatory regions of the EBV genome during latency in Akata cells revealed that both of the origins of lytic replication (OriLytL and OriLytR) and the key early lytic cycle promoters (*BZLF1*, *BRLF1*, *BMRF1*, and *BALF5*) show evidence of being associated with a repressive histone modification (H3K9me3) (Fig. 1). The data presented here identify a common occurrence of H3K9me3 association at several key lytic regulatory regions. In addition, a lower level of the repressive polycomb-associated histone modification (H3K27me3) was detected at these regions. Therefore, association with markers of repressive chromatin is a common feature of these early lytic regulatory regions. Surprisingly, these experiments also revealed that acetylated H3 was associated with these key regulatory regions, together with lower levels of acetylated H4.

We also questioned whether H3K9me3 and acetylated H3 are associated with late lytic genes, using BCRF1 and BNRF1 as examples. Both promoter regions have a similar high association of H3K9me3 and lower association with acetylated H3 as the early lytic promoters (Fig. 1F and G).

The interaction of both activation- and repression-associated chromatin with the early lytic regulatory regions could represent partially activated chromatin in latency, or it could result from a mixed cell population, with a few cells undergoing lytic cycle. To distinguish between these possibilities, we conducted sequential ChIP to determine whether the lytic regulatory regions in the EBV genome are associated with H3K9me3 and acetylated H3 simultaneously. The first round of precipitation with the H3K9me3 antibody showed substantial enrichment compared to the control antibody (Fig. 2A and B). Each batch of chromatin was then eluted and reprecipitated with either the H3K9me3 antibody or the acetylated H3 antibody. We investigated the association at the *BRLF1* promoter and a region adjacent to OriLytL. At both loci, only a small proportion of the H3K9me3 associated chromatin was found to coassociate with acetylated H3. In contrast, the con-

trol chromatin gave no signal with either antibody in the second round.

In the reverse experiment, the chromatin was first precipitated with the acetylated H3 antibody, or control, and subsequently with either the H3K9me3 antibody or the acetylated H3 antibody. In this case, the acetylated H3-associated chromatin was quantitatively reprecipitated with the H3K9me3 antibody (Fig. 3), suggesting that all of the acetylated H3 is associated with both histone marks. However, since only a minority of the DNA is associated with both histone marks and the presence of the activation-associated modification is known to be associated with lytic cycle (6, 7, 22–24, 32, 51), this probably represents the subpopulation of cells that spontaneously enters into the lytic cycle during normal growth of the Akata cell line. These data suggest that for the majority of cells in the latent population the lytic cycle regulatory regions are marked by the repressive histone modification H3K9me3.

Activation of H2AX associated with key lytic regulatory regions. Phosphorylation of H2AX occurs during lytic EBV replication in an inducible Zta expression system (29). We show here that there is little phosphorylation of H2AX during latency but that a substantial increase occurs in BL cells following entry into lytic cycle by stimulation of the B-cell receptor (Fig. 4A). Furthermore, it has been suggested that H2AX phosphorylation is important for the lytic cycle of the related gammaherpesvirus MHV68 (48, 50). We therefore sought to determine whether phosphorylated H2AX is associated with key early- and late-lytic-cycle regulatory regions and the latency control region Qp. This ChIP assessment of phosphorylated H2AX revealed negligible association of phosphorylated H2AX with the EBV genome during latency but a clear interaction with each of the key regulatory regions specifically during the lytic cycle (Fig. 4).

Phosphorylation of H2AX is associated with the DNA damage response pathway and phosphorylated H2AX form foci for the recruitment of repair components at sites of damaged DNA (30). We speculated that H2AX might become activated in response to the unusual DNA structures formed on the EBV genome during lytic replication. To question this, we stalled viral DNA replication using the drug acyclovir (stalled lytic cycle) and investigated whether association of the EBV genome with activated H2AX occurs. QPCR assays showed that acyclovir efficiently inhibited EBV genome replication (Fig. 5B) without altering the abundance of Zta or phosphorylated H2AX proteins (Fig. 5A). ChIP experiments revealed that even during stalled lytic cycle the phosphorylated H2AX associates with the EBV genome (Fig. 5C).

Since replication of the EBV genome is not required to stimulate phosphorylation of H2AX, this points to the action of a soluble enzyme rather than an enzyme that is tethered to DNA such as ATM. Further analysis of the location of phosphorylated H2AX revealed that it associates with the host genome during the lytic cycle (Fig. 5D and E). Blocking EBV genome replication with acyclovir does not prevent this increased association (Fig. 5F). Thus, the association of phosphorylated H2AX occurs equivalently with the host and EBV genomes. This global effect on H2AX points to the action of a soluble enzyme such as the EBV protein kinase (BGLF4), which is known to be able to promote phosphorylation of H2AX (48).

Association of Zta with key lytic regulatory regions on the EBV genome. We next explored the interaction of Zta with the lytic cycle regulatory regions during the early stages of the lytic cycle. We analyzed Zta binding *in vivo* during lytic replication using ChIP with a panel of amplicons to assess the interaction of

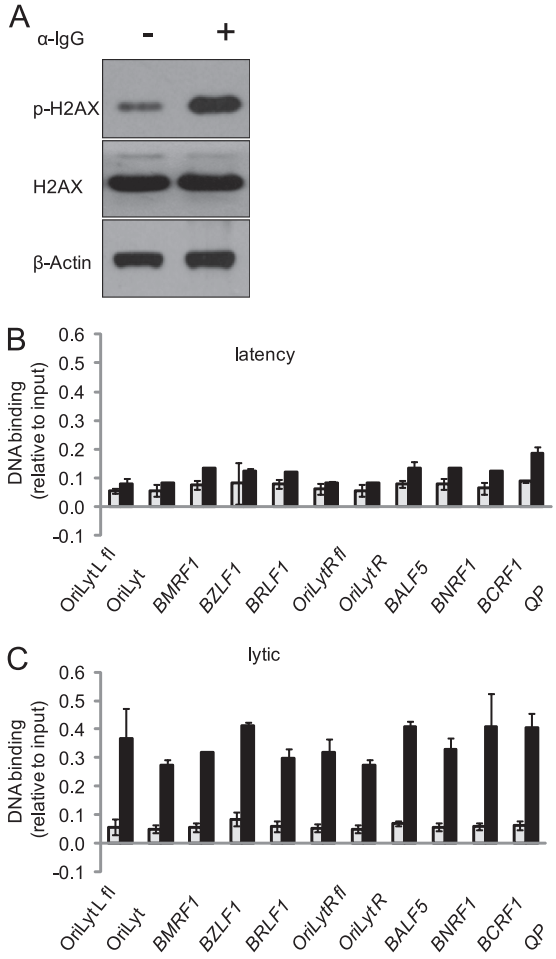

FIG 4 Association of phosphorylated H2AX with EBV genome. Akata cells were induced, or not, with anti-IgG. After 48 h, total protein extracts and chromatin were prepared. (A) Proteins were fractionated on SDS-polyacrylamide gels and subjected to Western blotting with the indicated antibodies. Lanes "+" and "–" refer to stimulation with IgG. (B and C) Chromatin was precipitated with a control antibody or an antibody that recognizes the phosphorylated form of H2AX.The associated DNA was purified and amplified with primers specific for each of the regulatory regions as indicated. The data from two independent batches of chromatin are expressed relative to the input chromatin with the standard error shown. The results for noninduced cells are shown in panel B, and the results for induced cells are shown in panel C. In each case, the gray bars represent the signal from the control antibody, and the black bars represent the signal from the phosphorylated H2AX antibody. The primers used to amplify each region were OriLytL flank (OL1), OriLyt (OL5), *BMRF1* promoter (BMRF1-2), *BZLF1* promoter (ZP4), *BRLF1* promoter (RP3), *BALF5* promoter (BALF5), and *BNRF1*, *BCRF1*, and the latency Qp promoter (QP).

Zta with both origins of lytic replication. This revealed a tight footprint of Zta binding over each of OriLytL and OriLytR (Fig. 6). It is expected that Zta would interact with OriLytL, since this has been seen in other cell lines (2). However, interaction of Zta with OriLytR has not been documented previously (Fig. 6). These data show that both OriLyts are recognized in an equivalent manner *in vivo* during lytic replication.

Analysis of the interaction between Zta and key early lytic cycle promoters revealed that Zta associates specifically with the promoters for *BZLF1* (Zp), *BRLF1* (Rp), and *BMRF1* (Fig. 7). Interestingly, the maximum binding to the Zp region (detected by ZP4

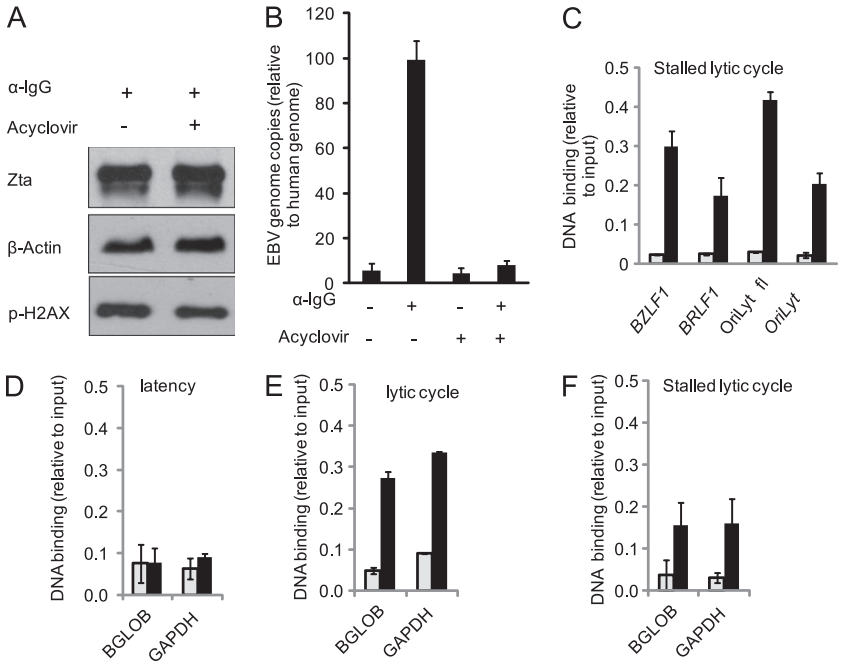

FIG 5 H2AX association with the EBV and host genomes during lytic cycle. Akata cells were induced or not with anti-IgG either in the presence or in the absence of acyclovir. After 48 h, total protein extracts, genomic DNA, and chromatin were prepared. Latency is represented by no IgG and no acyclovir, a stalled lytic cycle is represented by IgG and acyclovir, and the lytic cycle is represented by IgG with no acyclovir. (A) Proteins were fractionated on SDS-polyacrylamide gels and subjected to Western blotting with the indicated antibodies. Lanes "+" and "−" refer to stimulation with IgG and the presence of acyclovir. (B) Genomic DNA was subjected to QPCR for specific regions from the EBV (*BALF5*) and human (beta-globin) genes. The amount of EBV genome present is expressed relative to the amount of human gene, with the standard error shown. (C to F) Chromatin was precipitated with a control antibody or an antibody that recognizes the phosphorylated form of H2AX. The associated DNA was purified and amplified with primers specific for each of the EBV or human genome regulatory regions *β*-globin (BGLOB) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as indicated. The data from two independent batches of chromatin are expressed relative to the input chromatin, with the standard error shown. In each case, the gray bars represent the signal from the control antibody, and the black bars represent the signal from the phosphorylated H2AX antibody. The primer sets used to amplify each region were OriLytR flank (OR1), OriLyt (OL5), *BZLF1* promoter (ZP4), and *BRLF1* promoter (RP3).

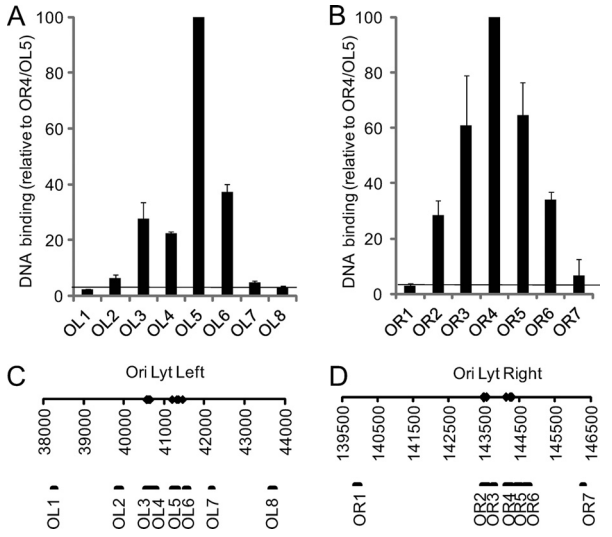

FIG 6 Zta association with the EBV origins of replication during lytic cycle. Akata cells were induced with anti-IgG where indicated. After 48 h, the chromatin was prepared. (A and B) Chromatin was precipitated with an antibody that recognizes Zta. The associated DNA was purified and amplified with primers sets specific for regions within OriLytL (A) or OriLytR (B). The data from two independent batches of chromatin are expressed relative to the maximal Zta signal at OriLyt, with the standard error shown. (C and D) The genomic region surrounding OriLytL and OriLytR are shown with the genome coordinates and the location of characterized ZREs (identified as diamonds). The amplicons generated by the primers sets are shown below.

primers) encompasses the ZIIIA and B sites, which can mediate autoactivation by Zta (17). It is intriguing that association with these promoters occurs to a lesser degree than the association of Zta with either lytic origin of replication. Indeed, the maximal binding at the *BZLF1* promoter (Zp) is only 17% of that are observed at OriLyt, and maximal binding at the *BRLF1* promoter (Rp) is only 7% of that at OriLyt (Fig. 7E).

Since the differential association between promoters and origins of replication could be connected to stabilization of Zta binding to the origin of lytic replication, as observed by El-Guindy et al. (10), we questioned whether there is any difference between the kinetics of the association of Zta with key early lytic promoters compared to the origins of lytic replication. Assessing binding across a time course during the first 48 h after the induction of lytic cycle revealed that the interaction between Zta and the key early lytic cycle regulatory regions occurs in an equivalent temporal manner detectable by 12 h postinduction (Fig. 8).

Zta coassociation with histone modifications. We hypothesize that several key early lytic cycle control regions of the EBV genome are associated with heterochromatin-like repressive chromatin during latency in Akata BL cells. Furthermore, once Zta is expressed, it associates with these control regions and promotes changes to the local chromatin environment. We tested this model by asking whether Zta coassociates with H3K9me3 during lytic cycle using sequential ChIP (Fig. 9). The first round of precipitation isolated Zta-bound chromatin and the second round inter-

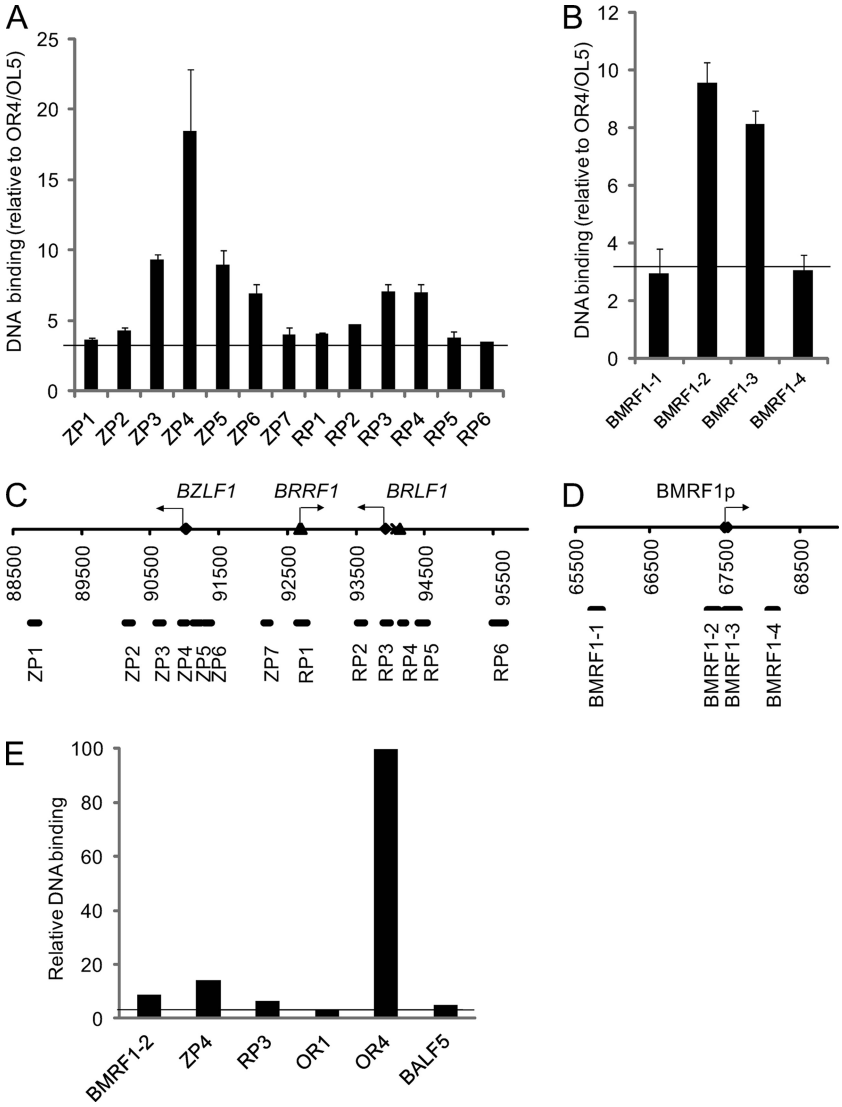

FIG 7 Zta association with key lytic EBV promoters during the lytic cycle. Akata cells were induced with anti-IgG where indicated. After 48 h, the chromatin was prepared. (A and B) Chromatin was precipitated with an antibody that recognizes Zta. The associated DNA was purified and amplified with primers sets specific for regions within the *BZLF1/BRLF1* promoter region (A) or the *BMRF1* promoter region (B). The data from two independent batches of chromatin are expressed relative to the maximal Zta signal at OriLyt, with the standard error shown. (C and D) The genomic regions surrounding the promoters are shown with the genome coordinates and the location of characterized ZREs (identified as diamonds). The amplicons generated by the primers sets are shown below. Transcription start sites and the direction of transcription are indicated by arrows. (E) The maximal signal for each region is expressed relative to maximal binding to the lytic origin (OR4).

rogated coassociation with H3K9me3. Parallel experiments with nonspecific antibodies established the validity of binding at each stage. Zta clearly associated with chromatin at the control regions in OriLyt, the *BZLF1* and *BRLF1* promoters in these experiments, whereas it did not associate with a region neighboring OrilytR (OR1) (Fig. 9). It is clear from these experiments that Zta is able to coassociate with H3K9me3 on DNA. Importantly, these experiments demonstrate that at three early EBV lytic cycle regulatory regions Zta is able to interact with the repressive chromatin environment that is characteristic of EBV lytic cycle regulatory regions during EBV latency.

We also investigated the coassociation of Zta with acetylated histone H3 48 h postinduction of lytic replication. Sequential ChIP revealed that approximately one-third of Zta is associated with acetylated histone 3 at this point (Fig. 10).

DISCUSSION

We propose a model in which key early lytic promoters and the origins of lytic replication are associated with a repressive heterochromatin-like environment during latency, which is overcome to allow expression of lytic cycle genes and origin function. This model is based on our analysis of the chromatin structure at key early lytic promoters and the origins of lytic replication and is also supported by a low-resolution genome-wide map of H3K9me3 association with the EBV genome in a different cell line (group I Mutu BL cells), recently published by Tempera et al. (49). The association of key lytic cycle regulatory regions with H3K9me3 in two BL cell lines derived from different lymphomas indicates that this association may be a common feature of EBV type I latency in BL cells.

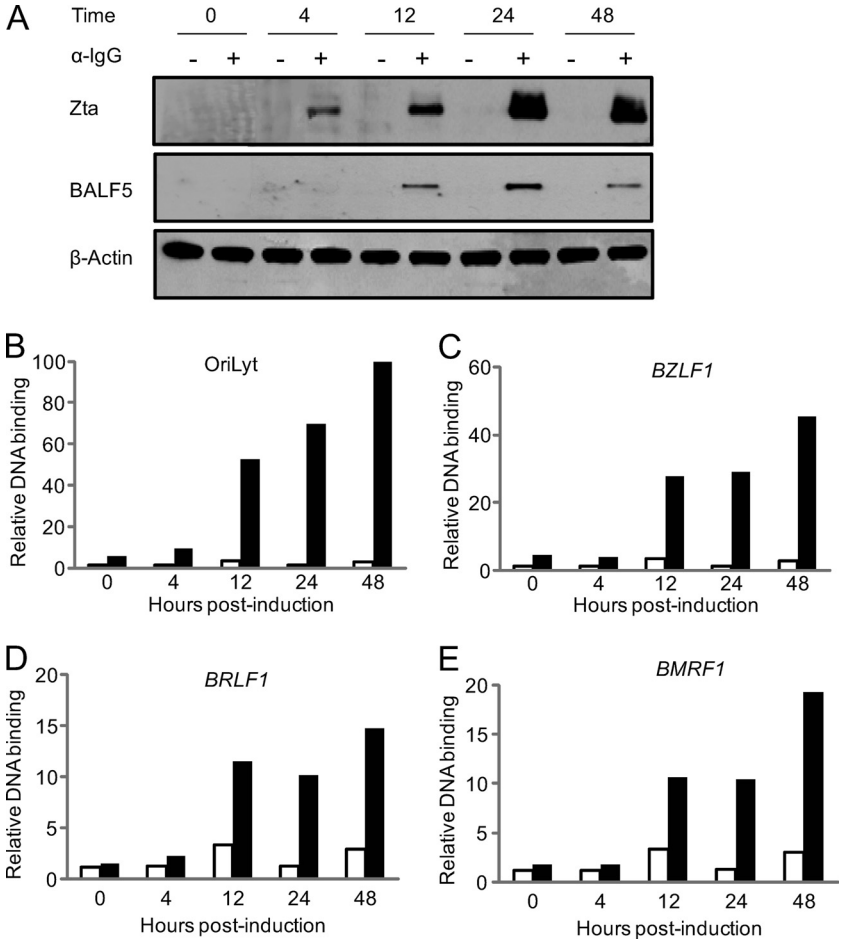

FIG 8 Zta association with EBV genome during the first 48 h of the lytic cycle. Akata cells were induced with anti-IgG where indicated. Total protein extracts and chromatin were prepared at 0, 4, 12, 24, and 48 h postinduction. (A) Proteins were fractionated on SDS-polyacrylamide gels and subjected to Western blotting with the indicated antibodies. Lanes "+" and "−" refer to stimulation with IgG, and the times (in hours) postinduction are indicated. (B to D) Chromatin was precipitated with an antibody that recognizes Zta. The associated DNA was purified and amplified with primers sets specific for regions at OriLyt (OL5) (B), the *BZLF1* promoter (ZP4) (C), the *BRLF1* promoter (RP3) (D), or the *BMRF1* promoter (BMRF1-2) (E). The data are expressed relative to the maximal Zta signal at OriLyt. In each case, the white bars represent the signal from chromatin from uninduced cells, and the black bars represent the signal from the chromatin from induced cells.

Several groups have shown that the *BZLF1* promoter is not associated with acetylated histones during latency but that the association increases significantly during lytic cycle (6, 23, 51). This is in agreement with the negligible level of histone H3 acetylation that we observed in the majority of cells during latency in Akata cells, which is followed by a strong coassociation of Zta with acetylated histone H3 during lytic cycle (Fig. 10).

In addition, we identified a further change to the chromatin landscape at these early lytic cycle regulatory regions: association with the activated form of the variant histone core protein H2AX. Activation of H2AX is associated with induction of double-strand breaks (38) and replication stress (12) and is seen during DNA fragmentation when cells undergo apoptosis (37). Activated H2AX plays a key role in the recruitment of DNA repair and signaling factors (35). H2AX activation and the subsequent partial activation of the DNA damage response during EBV lytic replication were documented by Kudoh et al. (29); we demonstrate here that the H2AX modification occurs at the key regulatory regions on the EBV genome during the lytic cycle, prior to EBV genome replication. There is a requirement for H2AX for the lytic cycle of the related virus MHV68 in some cell types, suggesting that it may contribute to gamma herpesvirus replication. Since it has not proved possible to knock down the expression of H2Ax completely, the impact that phosphorylation of H2AX has on EBV lytic cycle remains to be determined.

Zta is required for EBV lytic replication (13). It is a sequence-specific DNA-binding protein, with a promiscuous range of response elements; it can interact with at least 32 sequence variants of a 7-mer sequence (ZREs) (19). Zta has the unusual ability to recognize methylated DNA (2–4, 9, 26); indeed, some ZREs are entirely dependent on methylation for binding. This theoretically provides Zta with the ability to access DNA within the EBV genome even when it has been epigenetically silenced by DNA methylation. We show here that Zta interacts with both origins of lytic replication and with the promoters of *BZLF1*, *BRLF1*, and *BMRF1* in Akata cells at regions containing predicted ZREs (19). This supports and extends previous reports that Zta interacts with the EBV genome *in vivo* in other cell types (2, 9, 26, 52).

The presence of H3K9me3 correlates with repressive chromatin, which is considered to prevent transcription factors from in-

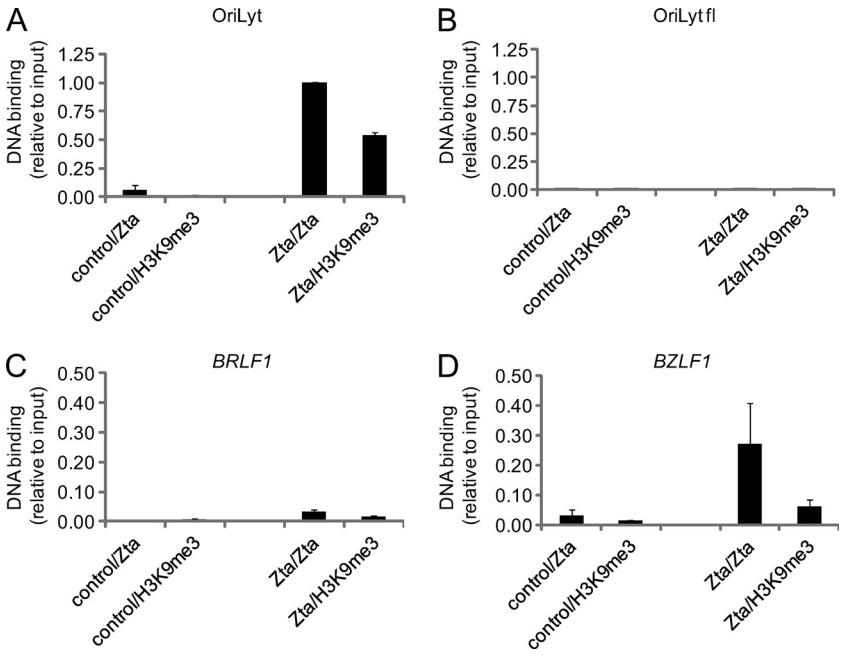

FIG 9 Zta coassociation with H3K9me3 during EBV lytic cycle. Akata cells were induced with anti-IgG. Chromatin was prepared at 48 h postinduction. The initial round of ChIP was undertaken with a Zta or a control antibody. The sample was then split, and either DNA was purified or the protein DNA complexes were eluted and subjected to a second-round of precipitation with either Zta or H3K9me3 antibodies. DNA was prepared from chromatin precipitated in the second round and was amplified with primers sets specific for regions at OriLyt (OL5), the region flanking OriLytR (OR1), the *BZLF1* (ZP4), or the *BRLF1* (RP3) promoters. The data from experiments carried out on two independent batches of chromatin are expressed relative to the binding of Zta to OR4.

teracting with DNA. Indeed, it was recently shown that the presence of repressive chromatin containing the H3K9me3 mark is sufficient to prevent the interaction of the p53 transcription factor with otherwise responsive promoters (44). It was not known previously what impact the association of Zta-responsive regulatory elements with H3K9me3 would have on the ability of Zta to bind to DNA. Using sequential ChIP we sought to determine whether Zta and H3K9me3 coexist on chromatin during the EBV lytic

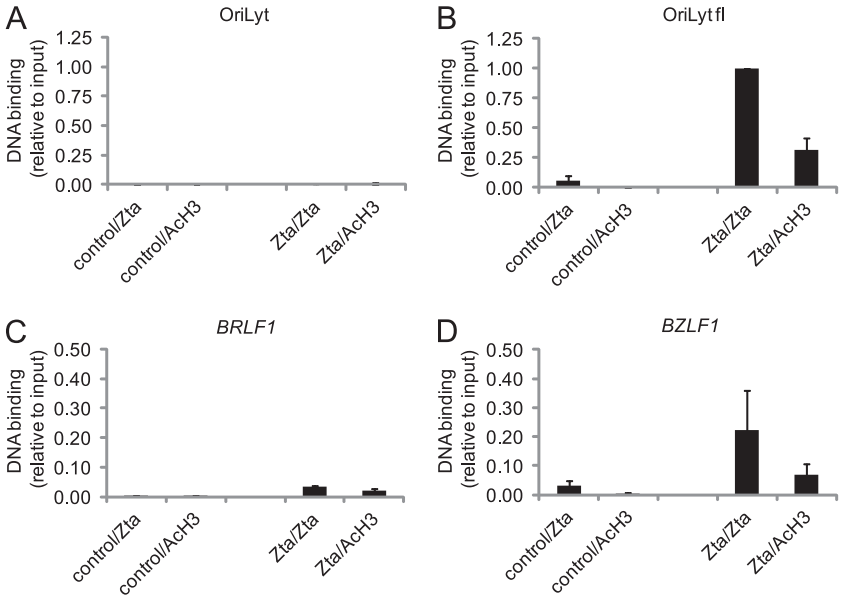

FIG 10 Zta coassociation with acetylated H3 during EBV lytic cycle. Akata cells were induced with anti-IgG. Chromatin was prepared at 48 h postinduction. The initial round of chromatin precipitation was undertaken with a Zta or a control antibody. The sample was then split, and either DNA was purified or the protein DNA complexes were eluted and subjected to a second round of precipitation with either Zta or acetylated H3 antibodies. DNA was prepared from chromatin precipitated in the second round and amplified with primers sets specific for regions at OriLyt (OL5), the region flanking OriLytR (OR1), the *BZLF1* (ZP4), or the *BRLF1* (RP3) promoters. The data from experiments carried out on two independent batches of chromatin are expressed relative to the binding of Zta to OR4.

cycle. This revealed that Zta clearly associated with chromatin containing the H3K9me3 modification providing proof that Zta is able to interact with lytic cycle regulatory regions even when they are encased in a repressive chromatin environment. Interestingly, methylated DNA is often associated with facultative heterochromatin and associated with H3K9me3. Therefore, Zta may have two routes to overcome epigenetic silencing of the EBV genome: (i) by its ability to coassociate with H3K9me3-bound chromatin and (ii) by its ability to interact with methylated ZREs.

ACKNOWLEDGMENT

This study was supported by a grant from the Wellcome Trust.

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